MUTATIONS OF THE PIK3CA GENE IN HUMAN CANCERS

[01] This application was made using funds provided by the United States government under grant nos. NIH-CA 62924 and NIH-CA 43460. The United States government therefore retains certain rights in the invention.

FIELD OF THE INVENTION

[02] The invention relates to the fields of diagnostic tests and therapeutic methods for cancer.

BACKGROUND OF THE INVENTION

[03] PI3Ks are lipid kinases that function as signal transducers downstream of cell surface receptors and mediate pathways important for cell growth, proliferation, adhesion, survival and motility (*1, 2*). Although increased PI3K activity has been observed in many colorectal and other tumors (*3, 4*), no intragenic mutations of PI3K have been identified.

[04] Members of the PIK3 pathway have been previously reported to be altered in cancers, for example, the PTEN tumor suppressor gene (*15, 16*), whose function is to reverse the phosphorylation mediated by PI3Ks (*17, 18*). Reduplication or amplification of the chromosomal regions containing PIK3CA and AKT2 has been reported in some human cancers (*2, 19, 20*), but the genes that are the targets of such large-scale genetic events have not been and cannot easily be defined.

BRIEF SUMMARY OF THE INVENTION

[05] In a first embodiment a method is provided for assessing cancer in a human tissue suspected of being cancerous of a patient. A non-synonymous,

intragenic mutation in a PIK3CA coding sequence is detected in a body sample of a human suspected of having a cancer. The human is identified as likely to have a cancer if a non-synonymous, intragenic mutation in PIK3CA coding sequence is determined in the body sample.

[06] In a second embodiment of the invention a method is provided for inhibiting progression of a tumor in a human. An antisense oligonucleotide or antisense construct is administered to a tumor. The antisense oligonucleotide or RNA transcribed from the antisense construct is complementary to mRNA transcribed from PIK3CA. The amount of p110α protein expressed by the tumor is thereby reduced.

[07] Another embodiment of the invention provides a method of inhibiting progression of a tumor in a human. siRNA comprising 19 to 21 bp duplexes of a human PIK3CA mRNA with 2 nt 3' overhangs are administered to the human. One strand of the duplex comprises a contiguous sequence selected from mRNA transcribed from PIK3CA (SEQ ID NO: 2). The amount of p110α protein expressed by the tumor is thereby reduced.

[08] According to another aspect of the invention a method is provided for inhibiting progression of a tumor. A molecule comprising an antibody binding region is administered to a tumor. The antibody binding region specifically binds to PIK3CA (SEQ ID NO: 3).

[09] Another embodiment of the invention provides a method of identifying candidate chemotherapeutic agents. A wild-type or activated mutant p110α (SEQ ID NO: 3) is contacted with a test compound. p110α activity is then measured. A test compound is identified as a candidate chemotherapeutic agent if it inhibits p110α activity.

[10] Still another embodiment of the invention is a method for delivering an appropriate chemotherapeutic drug to a patient in need thereof. A non-synonymous, intragenic mutation in a PIK3CA coding sequence (SEQ ID NO:

1) is determined in a test tissue of a patient. A p110α inhibitor is administered to the patient.

[11] An additional aspect of the invention provides a set of one or more primers for amplifying and/or sequencing PIK3CA. The primers are selected from the group consisting of forward primers, reverse primers and sequencing primers. The forward primers are selected from the group consisting of: SEQ ID NO: 6 to 158; the reverse primers are selected from the group consisting of: SEQ ID NO: 159 to 310; and the sequencing primers are selected from the group consisting of: SEQ ID NO: 311 to 461.

BRIEF DESCRIPTION OF THE DRAWINGS

[12] Fig. 1. Detection of mutations in of PIK3CA. Representative examples of mutations in exons 9 and 20. In each case, the top sequence chromatogram was obtained from normal tissue and the three lower sequence chromatograms from the indicated tumors. Arrows indicate the location of missense mutations. The nucleotide and amino acid alterations are indicated above the arrow.

[13] Fig. 2. Distribution of mutations in PIK3CA. Arrows indicate the location of missense mutations, and boxes represent functional domains (p85BD, p85 binding domain; RBD, Ras binding domain; C2 domain; Helical domain; Kinase domain). The percentage of mutations detected within each region in cancers is indicated below.

[14] Figs. 3A-3C. Increased lipid kinase activity of mutant p110α. NIH3T3 cells were transfected with empty vector or with vector constructs containing either wild-type p110α or mutant p110α (H1047R) as indicated above the lanes. Immunoprecipitations were performed either with control IgG or anti-p85 polyclonal antibodies. (Fig. 3A) Half of the immunoprecipitates were subjected to a PI3-kinase assay using phosphatidylinositol as a substrate. "PI3P" indicates the position of PI-3-phosphate determined with standard phosphatidyl markers and "Ori" indicates the origin. (Fig. 3B) The other half

of the immunoprecipitates was analyzed by western blotting with anti-p110α antibody. (Fig. 3C) Cell lysates from transfected cells contained similar amounts of total protein as determined by western blotting using an anti-α-tubulin antibody. Identical results to those shown in this figure were observed in three independent transfection experiments.

DETAILED DESCRIPTION OF THE INVENTION

[15] The clustering of mutations within PIK3CA make it an excellent marker for early detection or for following disease progression. Testing focused in the clustered regions will yield most of the mutant alleles.

[16] The human PIK3CA coding sequence is reported in the literature and is shown in SEQ ID NO: 1. This is the sequence of one particular individual in the population of humans. Humans vary from one to another in their gene sequences. These variations are very minimal, sometimes occurring at a frequency of about 1 to 10 nucleotides per gene. Different forms of any particular gene exist within the human population. These different forms are called allelic variants. Allelic variants often do not change the amino acid sequence of the encoded protein; such variants are termed synonymous. Even if they do change the encoded amino acid (non-synonymous), the function of the protein is not typically affected. Such changes are evolutionarily or functionally neutral. When human PIK3CA is referred to in the present application all allelic variants are intended to be encompassed by the term. The sequence of SEQ ID NO: 1 is provided merely as a representative example of a wild-type human sequence. The invention is not limited to this single allelic form of PIK3CA. For purposes of determining a mutation, PIK3CA sequences determined in a test sample can be compared to a sequence determined in a different tissue of the human. A difference in the sequence in the two tissues indicates a somatic mutation. Alternatively, the sequence determined in a PIK3CA gene in a test sample can be compared to the sequence of SEQ ID NO: 1. A difference between the test sample

sequence and SEQ ID NO: 1 can be identified as a mutation. Tissues suspected of being cancerous can be tested, as can body samples that may be expected to contain sloughed-off cells from tumors or cells of cancers. Suitable body samples for testing include blood, serum, plasma, sputum, urine, stool, nipple aspirate, saliva, and cerebrospinal fluid.

[17] Mutations in PIK3CA cluster in exons 9 (SEQ ID NO: 4) and 20 (SEQ ID NO: 5). Other mutations occur, but these two exons appear to be the hotspots for mutations. Many mutations occur in PIK3CA's helical domain (nt 1567-2124 of SEQ ID NO: 2) and in its kinase domain (nt 2095-3096 of SEQ ID NO: 2). Fewer occur in PIK3CA's P85BD domain (nt 103-335 of SEQ ID NO: 2). Mutations have been found in exons 1, 2, 4, 5, 7, 9, 13, 18, and 20. Any combination of these exons can be tested, optionally in conjunction with testing other exons. Testing for mutations can be done along the whole coding sequence or can be focused in the areas where mutations have been found to cluster. Particular hotspots of mutations occur at nucleotide positions 1624, 1633, 1636, and 3140 of PIK3CA coding sequence.

[18] PIK3CA mutations have been found in a variety of different types of tumors. Thus any of a variety of tumors can be tested for PIK3CA mutations. These tissues include, without limitation: colorectal tissue, brain tissue, gastric tissue, breast tissue, and lung tissue.

[19] Any type of intragenic mutation can be detected. These include substitution mutations, deletion mutations, and insertion mutations. The size of the mutations is likely to be small, on the order of from 1 to 3 nucleotides. Mutations which can be detected include, but are not limited to G1624A, G1633A, C1636A, A3140G, G113A, T1258C, G3129T, C3139T, and G2702T. Any combination of these mutations can be tested.

[20] The mutations that are found in PIK3CA appear to be activating mutations. Thus therapeutic regimens involving inhibition of p110α activity or expression can be used to inhibit progression of a tumor in a human.

Inhibitory molecules which can be used include antisense oligonucleotides or antisense constructs, a molecule comprising an antibody binding region, and siRNA molecules. Molecules comprising an antibody binding region can be full antibodies, single chain variable regions, antibody fragments, antibody conjugates, etc. The antibody binding regions may but need not bind to epitopes contained within the kinase domain (nt 2095-3096 of SEQ ID NO: 2) of PIK3CA, the helical domain (nt 1567-2124 of SEQ ID NO: 2) of PIK3CA, or the P85BD domain (nt 103-335 of SEQ ID NO: 2) of PIK3CA.

[21] Antisense constructs, antisense oligonucleotides, RNA interference constructs or siRNA duplex RNA molecules can be used to interfere with expression of PIK3CA. Typically at least 15, 17, 19, or 21 nucleotides of the complement of PIK3CA mRNA sequence are sufficient for an antisense molecule. Typically at least 19, 21, 22, or 23 nucleotides of PIK3CA are sufficient for an RNA interference molecule. Preferably an RNA interference molecule will have a 2 nucleotide 3' overhang. If the RNA interference molecule is expressed in a cell from a construct, for example from a hairpin molecule or from an inverted repeat of the desired PIK3CA sequence, then the endogenous cellular machinery will create the overhangs. siRNA molecules can be prepared by chemical synthesis, in vitro transcription, or digestion of long dsRNA by Rnase III or Dicer. These can be introduced into cells by transfection, electroporation, or other methods known in the art. See Hannon, GJ, 2002, RNA Interference, *Nature* 418: 244-251; Bernstein E et al., 2002, The rest is silence. *RNA 7:* 1509-1521; Hutvagner G et al., RNAi: Nature abhors a double-strand. *Curr. Opin. Genetics & Development 12:* 225-232; Brummelkamp, 2002, A system for stable expression of short interfering RNAs in mammalian cells. *Science 296:* 550-553; Lee NS, Dohjima T, Bauer G, Li H, Li M-J, Ehsani A, Salvaterra P, and Rossi J. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nature Biotechnol.* **20**:500-505; Miyagishi M, and Taira K. (2002). U6-promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress

targeted gene expression in mammalian cells. *Nature Biotechnol.* **20**:497-500; Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, and Conklin DS. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Dev.* **16**:948-958; Paul CP, Good PD, Winer I, and Engelke DR. (2002). Effective expression of small interfering RNA in human cells. *Nature Biotechnol.* **20**:505-508; Sui G, Soohoo C, Affar E-B, Gay F, Shi Y, Forrester WC, and Shi Y. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99(6)**:5515-5520; Yu J-Y, DeRuiter SL, and Turner DL. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99(9)**:6047-6052.

[22] Antisense or RNA interference molecules can be delivered *in vitro* to cells or *in vivo, e.g.,* to tumors of a mammal. Typical delivery means known in the art can be used. For example, delivery to a tumor can be accomplished by intratumoral injections. Other modes of delivery can be used without limitation, including: intravenous, intramuscular, intraperitoneal, intraarterial, local delivery during surgery, endoscopic, subcutaneous, and *per os.* In a mouse model, the antisense or RNA interference can be adminstered to a tumor cell *in vitro*, and the tumor cell can be subsequently administered to a mouse. Vectors can be selected for desirable properties for any particular application. Vectors can be viral or plasmid. Adenoviral vectors are useful in this regard. Tissue-specific, cell-type specific, or otherwise regulatable promoters can be used to control the transcription of the inhibitory polynucleotide molecules. Non-viral carriers such as liposomes or nanospheres can also be used.

[23] Using the p110α protein according to the invention, one of ordinary skill in the art can readily generate antibodies which specifically bind to the proteins. Such antibodies can be monoclonal or polyclonal. They can be chimeric, humanized, or totally human. Any functional fragment or derivative of an

antibody can be used including Fab, Fab', Fab2, Fab'2, and single chain variable regions. So long as the fragment or derivative retains specificity of binding for the endothelial marker protein it can be used. Antibodies can be tested for specificity of binding by comparing binding to appropriate antigen to binding to irrelevant antigen or antigen mixture under a given set of conditions. If the antibody binds to the appropriate antigen at least 2, 5, 7, and preferably 10 times more than to irrelevant antigen or antigen mixture then it is considered to be specific.

[24] Techniques for making such partially to fully human antibodies are known in the art and any such techniques can be used. According to one particularly preferred embodiment, fully human antibody sequences are made in a transgenic mouse which has been engineered to express human heavy and light chain antibody genes. Multiple strains of such transgenic mice have been made which can produce different classes of antibodies. B cells from transgenic mice which are producing a desirable antibody can be fused to make hybridoma cell lines for continuous production of the desired antibody. See for example, Nina D. Russel, Jose R. F. Corvalan, Michael L. Gallo, C. Geoffrey Davis, Liise-Anne Pirofski. Production of Protective Human Antipneumococcal Antibodies by Transgenic Mice with Human Immunoglobulin Loci *Infection and Immunity* April 2000, p. 1820-1826; Michael L. Gallo, Vladimir E. Ivanov, Aya Jakobovits, and C. Geoffrey Davis. The human immunoglobulin loci introduced into mice: V (D) and J gene segment usage similar to that of adult humans *European Journal of Immunology* 30: 534-540, 2000; Larry L. Green. Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies *Journal of Immunological Methods 231* 11-23, 1999; Yang X-D, Corvalan JRF, Wang P, Roy CM-N and Davis CG. Fully Human Anti-interleukin-8 Monoclonal Antibodies: Potential Therapeutics for the Treatment of Inflammatory Disease States. *Journal of Leukocyte Biology* Vol. 66, pp401-410 (1999); Yang X-D,

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[25] Antibodies can also be made using phage display techniques. Such techniques can be used to isolate an initial antibody or to generate variants with altered specificity or avidity characteristics. Single chain Fv can also be used as is convenient. They can be made from vaccinated transgenic mice, if desired. Antibodies can be produced in cell culture, in phage, or in various animals, including but not limited to cows, rabbits, goats, mice, rats, hamsters, guinea pigs, sheep, dogs, cats, monkeys, chimpanzees, apes.

[26] Antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. Such labeled antibodies can be used for diagnostic techniques, either *in vivo*, or in an isolated test sample.

Antibodies can also be conjugated, for example, to a pharmaceutical agent, such as chemotherapeutic drug or a toxin. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 (^{131}I), yttrium-90 (^{90}Y), bismuth-212 (^{212}Bi), bismuth-213 (^{213}Bi), technetium-99m (^{99m}Tc), rhenium-186 (^{186}Re), and rhenium-188 (^{188}Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from chinese cobra (naja naja atra), and gelonin (a plant toxin); ribosome inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by *Aspergillus restrictus*), saporin (a ribosome inactivating protein from *Saponaria officinalis*), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing antitumor agents (*e.g.*, antisense oligonucleotides, plasmids which encode for toxins, methotrexate, etc.); and other antibodies or antibody fragments, such as F(ab).

[27] Those of skill in the art will readily understand and be able to make such antibody derivatives, as they are well known in the art. The antibodies may be cytotoxic on their own, or they may be used to deliver cytotoxic agents to particular locations in the body. The antibodies can be administered to individuals in need thereof as a form of passive immunization.

[28] Given the success of small molecule protein kinase inhibitors, one can develop specific or non-specific inhibitors of p110α for treatment of the large number of patients with these mutations or cancers generally. It is clearly possible to develop broad-spectrum PI3K inhibitors, as documented by studies of

LY294002 and wortmannin (*2, 21,22*). Our data suggest that the development of more specific inhibitors that target p110α but not other PI3Ks would be worthwhile.

[29] Candidate chemotherapeutic agents can be identified as agents which inhibit p110α activity or expression. Test compounds can be synthetic or naturally occurring. They can be previously identified to have physiological activity or not. Tests on candidate chemotherapeutic agents can be run in cell-free systems or in whole cells. p110α activity can be tested by any means known in the art. These include methods taught in references 2, 22 and in Truitt et al., *J. Exp. Med.*, 179, 1071-1076 (1994). Expression can be monitored by determining PI3KCA protein or mRNA. Antibody methods such as western blotting can be used to determine protein. Northern blotting can be used to measure mRNA. Other methods can be used without limitation. When testing for chemotherapeutic agents, the p110α used in the assay can be a wild-type or an activated form. The activated form may contain a substitution mutation selected from the group consisting of E542K, E545K, Q546K, and H1047R. Moreover, inhibitors can be tested to determine their specificity for either p110α or an activated form of p110α . Comparative tests can be run against similar enzymes including PIK3CB, PIK3CG, PIK3C2A, PIK3C2B, PIK3C2G, PIK3C3, A-TM, ATR, FRAP1, LAT1-3TM,SMG1, PRKDC, and TRRAP to determine the relative specificity for the p110α enzyme.

[30] Once a non-synonymous, intragenic mutation in a PIK3CA coding sequence is identified in a test tissue of a patient, that information can be used to make therapeutic decisions. Patients with such mutations are good candidates for therapy with a p110α inhibitor. Such inhibitors can be specific or general for the family of inhibitors. Such inhibitors include LY294002 and wortmannin. Such inhibitors further include molecules comprising an antibody binding region specific for p110α . Such molecules are discussed above.

[31] Sets of primers for amplifying and/or sequencing PIK3CA can be provided in kits or assembled from components. Useful sets include pairs of forward and reverse primers optionally teamed with sequencing primers. The forward primers are shown in SEQ ID NO: 6 to 158. The reverse primers are shown in SEQ ID NO: 159 to 310. The sequencing primers are shown in : SEQ ID NO: 311 to 461. Pairs or triplets or combinations of these pairs or triplets can be packaged and used together to amplify and/or sequence parts of the PIK3CA gene. Pairs can be packaged in single or divided containers. Instructions for using the primers according to the methods of the present invention can be provided in any medium which is convenient, including paper, electronic, or a world-wide web address.

[32] While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the spirit and scope of the invention as set forth in the appended claims.

EXAMPLES

Example 1—This example demonstrates that the PIK3CA gene is the predominant target of mutations in this gene family

[33] To evaluate whether PI3Ks is genetically implicated in tumorigenesis, we directly examined the DNA sequences of members of this gene family in colorectal cancers.

[34] PI3K catalytic subunits are divided into three major classes depending on their substrate specificity (*5*). Additionally, a set of more distantly related proteins, including members of the mTOR family, constitute a fourth class (*6*). We used Hidden Markov models to identify 15 human genes containing kinase domains related to those of known PI3Ks in the human genome (*7*). These

comprised seven PI3Ks, six members of the mTOR subfamily and two uncharacterized PI3K-like genes (Table 1).

Table 1. PI3K genes analyzed

Gene name	Celera Accession	Genbank Accession	Alternate names	Group*
PIK3CA	hCT1640694	NM_006218	p110-alpha	Class IA
PIK3CB	hCT7084	NM_006219	PIK3C1, p110-beta	Class IA
PIK3CD	hCT2292011	NM_005026	p110-delta	Class IA
PIK3CG	hCT7976	NM_002649	PI3CG, PI3K-gamma	Class IB
PIK3C2A	hCT2270768	NM_002645	CPK, PI3-K-C2A, PI3K-C2alpha	Class II
PIK3C2B	hCT7448	NM_002646	C2-PI3K, PI3K-C2beta	Class II
PIK3C2G	hCT1951422	NM_004570	PI3K-C2-gamma	Class II
PIK3C3	hCT13660	NM_002647	Vps34	Class III
ATM	hCT29277	NM_000051	AT1, ATA, ATC, ATD, ATE, ATDC	Class IV
ATR	hCT1951523	NM_001184	FRP1, SCKL, SCKL1	Class IV
FRAP1	hCT2292935	NM_004958	FRAP, MTOR, FRAP2, RAFT1, RAPT1	Class IV
SMG1	hCT2273636	NM_014006	ATX, LIP, KIAA0421	Class IV
PRKDC	hCT2257127	NM_006904	p350, DNAPK, DNPK1, HYRC1, XRCC7	Class IV
TRRAP	hCT32594	NM_003496	TR-AP, PAF400	Class IV
none	hCT2257641	none		Class IV
none	hCT13051	none		Class IV

*PI3K genes are grouped into previously described classes (S3,S4). Class I, II and III comprise PI3K catalytic subunits, while class IV comprises PI3K-like genes including members of the mTOR (target of rapamycin), ATM (ataxia telangiectasia mutated), and DNAPK (DNA-dependent protein kinase) subfamilies, as well as two previously uncharacterized genes.

[35] We initially examined 111 exons encoding the predicted kinase domains of these genes (Table 2). The exons were polymerase chain reaction (PCR) amplified and directly sequenced from genomic DNA of 35 colorectal cancers (*8*). Only one of the genes (PIK3CA) contained any somatic (*i.e.,* tumor-specific) mutations.

Table 2. Primers used for PCR amplification and sequencing

Gene and Exon Name	Forward Primer[1]	Reverse Primer[2]	Sequencing Primer[3]
hCT2270768-Ex21	TTCCAGCCTGGGTAACAAAG	CGTCAGAACAAGACCCTGTG	AAAGGGGAAATGCGTAGGAC
hCT2270768-Ex22	CCTGACCTCAGGTGTTCTGC	CCCGGCCACTAAGTTATTTTTC	TCCCAAAGTGCTGGGATTAC
hCT2270768-Ex23	TGCACATTCTGCACGTGTATC	CTGCCATTAAATGCGTCTTG	CCAGAACTTAAAGTGAAATTTAAAAAG
hCT2270768-Ex24	TCCCAGTTTGTATGCTATTGAGAG	CTTTGGGCCTTTTTCATTCC	GCGAGGCAAAACACAAAGC
hCT2270768-Ex25	TGGAAATTCAAAAGTGTGTGG	TGTCTGGCTTATTTCACACG	TTGGAAATGGCTGTACCTCAG
hCT2270768-Ex26	CACTAATGAACCCCTCAAGACTG	AACTTTTGACAGCCTACTATGTGC	TACTTGAGCAGCCCACAGG
hCT2270768-Ex 27- 1	TCCTTGGCAAAGTGACAATC	GACCATTCATGAAAGAAACAAGC	AAAGGAATGAAAGTGGTTTTTGTC
hCT13660-Ex16	CTCTCACATACAACACCATCTCC	CCATGTACCGGTAACAAAAGAAG	TGCAATGTAATAGTTTTCCAAGG
hCT13660-Ex17	ATGTATCTCATTGAAAACCCAAC	TGAGCTTTCTAGGATCGTACCTG	CAGCAAATGAACTAAGCCACAG
hCT13660-Ex18	TCCCAAAGTGCTGGGATTAC	GCAGGAAGGTCCAACTTGTC	TGCTATACTATTTGCCCACAAAAC
hCT13660-Ex19	CCTATGACATAAATGCCAGTACAAAC	ATCTTCAACTGCGAACATGC	GAATGCATTTATTCAGAGATGAGG
hCT13660-Ex20	TCTTTTGTTCAGTCAGCATCTCTC	AAGCATCAATGACTACTTTAATCAAC	TGCTAGACACTTGCTGGTCAC
hCT13660-Ex21	TTGAGAATTCAGATGAGAAACCAG	TCCCAAAGTGCTGGGATTAC	TTGATATTAAAGTTGCACAAACTGC
hCT13660-Ex22	GAAGGCCACTCTCAAACCTG	TTGTTGCCTTTGTCATTTTG	TCAATTGTGTGACATATCACCTACC
hCT13660-Ex23	TCAAGGCTTGCATTTCATTG	ATGTGACTGTGGGCAGGAAC	TCACTGTAGAAATCCAAGTACCAC
hCT13660-Ex24	TTCCACACTCCAAAGAATGC	GCTGGTGAGATGTCAAAACG	TCTGCATCAGTTTGATTCTGC
hCT13660-Ex 25- 1	AATTGCAATCCTCTTGGTAGC	TCAACATATTACTTCCTCCAGAACTC	AATGCACTTTTTATTTTATTAG
hCT32594-Ex 66- 2	GCCAAGACCAAGCAACTCC	TTCTCCCATGTCAGGGAATC	GAAAAGTGCCGGTTCTTGAG
hCT32594-Ex 67- 1	ATAAACGACCGCTGGCCTAC	GACCCTCAAAGGCTAACGTG	GCCTACACAGTCCGTTTTCC
hCT32594-Ex 67- 2	GTACATCCGGGGACACAATG	TCCCTGGTCAGCACAGACTAC	AGAGGAGCGTGTGTTGCAG
hCT32594-Ex68	ACCGGGTTCTTCCAGCTAAG	AGCTGTCTCATTTCCACCATC	ACTCTGACGGTGGAGCTGAG
hCT32594-Ex 69- 1	CAATGCGTGCGTTAAATCTG	CGCGTCGTTTATGTCAAATC	GCTCTTGGTGCTAAGTTAAAGAGG

Table 2. Primers used for PCR amplification and sequencing

hCT32594-Ex 69- 2	CCCAATGCCACGGACTAC	CGCGTCGTTTATGTCAAATC	ATCCAGCTGGCTCTGATAGG
hCT32594-Ex70	ATCCAGCTGGCTCTGATAGG	CATAACACACAGGGGTGCTG	TGAACAGCCAGATCCTCTCC
hCT32594-Ex71	CTGGTGCTGAAACTCGACTG	GAACTGGGCGAGGTTGTG	GTCCCACCTTGTTAGGAAGC
hCT32594-Ex 72- 1	GTCTCGTTCTCTCCCTCACG	TCCCTTTCTTACACGCAAAC	TGGCATTCTGAAAACGGTTC
hCT32594-Ex 72- 2	CACAACCTCGCCCAGTTC	CAGTTCCGCCTGTACATTCAC	GCAAACAGCCTGGACAATC
hCT7976-Ex5	AGCATCACCCTCAGAGCATAC	AGCGCTCCTGCTTTCAGTC	CACATATTTCTGTCCCCTGTTG
hCT7976-Ex6	TGCCATACCTCTTAGGCACTTC	GTCTTGGCGCAGATCATCAC	TGTGGTTCTTTGGAGCACAG
hCT7976-Ex7	CGACAGAGCAAGATTCCATC	TTTTGTCACCAGTTGAAATGC	CCAAGGTACATTTCGGAAAAC
hCT7976-Ex8	AGATTGCCATCTGAGGAAGG	GACTGGGAAAAAGCATGAGC	ACCAGCCCTTTCCTCTTGTC
hCT7976-Ex9	GCATGGAGAGGAAGTGAACC	CGGTGATCATAATATTGTCATTGTG	TTCTTCCTCATGCCATTGTG
hCT7976-Ex10	TGGCCAGAGAGTTTGATTTATG	GGAAGTGTGGGCTTGTCTTC	GTGGCATCTGGCTGTCATC
hCT7976-Ex 11- 1	CCCTCAATCTCTTGGGAAAG	TGCACAGTCCATCCTTTGTC	CAATTAGTTTTCCTTGAGCACTCC
hCT7976-Ex 11- 2	TGGTTTCTTCTCATGGACAGG	AATGCCAGCTTTCACAATGTC	TCTTCTTTATCCAGGACATCTGTG
hCT7448-Ex21	GGGTGTCCACACTTCTCAGG	GGCCAAGACCACATGGTAAG	CCTGGGAGAGGTCTGGTTC
hCT7448-Ex22	CCGGAAGAAACAATGAGCAG	TCCTACATTAAGACAGCATGGAAC	GGCAGCATCTTGGTCTGAAG
hCT7448-Ex23	GGTGTGAGCTGAGTGAGCAG	TGCCTCCCTTTTAAGGCTATC	GAGCACTTGGGAGACCTGAG
hCT7448-Ex24	GTGGGAATGACCTTCCTTTC	AGGTCCTTCTGCCAACAAAG	AGGGAAGCATGAGCACAGTC
hCT7448-Ex25	GGATGAACAGGCAGATGTGAG	CGTCTTCTCTCCTCCAATGC	TGAGTTCTGTCTGGCTGTGG
hCT7448-Ex26	AGCCCCTTCTATCCAGTGTG	GGTATTCAGTTGGGGCTCAG	TGATGAGGGATGAGGGAAAC
hCT7448-Ex27	TGCCCACAGCATCTGTCTAC	TGTATCCACGTGGTCAGCTC	AGGGTTAGGGAGCCTAGCTG
hCT7448-Ex 28- 1	ATTGTGTGCCAGTCATTTGC	ACAGGACGCTCGGTCAAC	TCCTTGGAACACCCCTGTC
hCT1951523-Ex 39- 2	TTCCACATTAAGCATGAGCAC	TTGCCATCAGTACAAATGAGTTTAG	CAGTCATGATACCTACACTTCCATC
hCT1951523-Ex40	GACAGTCATTCTTTTCATAGGTCATAG	TTCCTGCTTTTTAAGAGTGATCTG	CAACTCTGAAATAAAAGCAATCTGG
hCT1951523-Ex41	CCACATAGTAAGCCTTCAATGAC	AGGAAGGAAGGGATGGAAAC	TTCTTTGGTTATGAAATGAACAATC
hCT1951523-Ex42	TGAAAAATGTTCCTTTATTCTTG	AGAAACCACTCATGAAAA	TTGAATAAAAGTAGATGTTTCTTGTCC
hCT1951523-Ex43	TCTGAGAACATTCCCTGATCC	CGCATTACTACATGATCCACTG	TACCAAGAATATAATACGTTGTTATGG
hCT2257127-Ex76	TCAGCTCTCTAATCCTGAACTGC	TGTCACAGAAAGCATGAGACC	CGGCTTCTGGCACATAAAAC
hCT2257127-Ex 77- 1	AGCAGAGAAGAAACATATACCAT	AGAAATAACTGTCAATATCCCAGTATCAC	CCATTGAGCACTCCATTCATTAC
hCT2257127-Ex 77- 2	CATTTTGGGAAAGGAGGTTC	TCATTAAACATTTAGTAATGTGTGCTC	CCCTGGGAATCTGAAAGAATG

Table 2. Primers used for PCR amplification and sequencing

hCT2257127-Ex78	ATTACAGGCGTGAGCCACTG	AGGCAACAGGGCAAGACTC	TGGGCCGTTGTCTCATATAC
hCT2257127-Ex 79- 1	TTTGGCACTGTCTTCAGAGG	CCTGAAAGGGAGAATAAAAGG	CACTCTGGCTTTTCCCTCTG
hCT2257127-Ex 79- 2	AGAGGGAACACCCTTTCCTG	CCTGAAAGGGAGAATAAAAGG	AGGTCATGAATGGGATCCTG
hCT2257127-Ex80	TATAGCGTTGTGCCCATGAC	TATTGACCCAGCCAGCAGAC	CATATTGCTTGGCGTCCAC
hCT2257127-Ex81	TCCTGCCTCTTTGCTATTTTTCAATG	TATATTGAGACTCAAATATCGA	TCTTGGTGATCTTTGCCTTTG
hCT2257127-Ex82	TTGCCTCAGAGAGATCATCAAG	TGATGCATATCAGAGCGTGAG	TCATCAAGATTATTCGATATTTGAGTC
hCT2257127-Ex 83- 1	TAGGGGCGCTAATCGTACTG	TTCAATGACCATGACAAAACG	CGAGAAAGTAAAGTGCCTGCTG
hCT2257127-Ex 83- 2	TCTGATATGCATCAGCCACTG	TTCAATGACCATGACAAAACG	CGGGATTGGAGACAGACATC
hCT2257127-Ex84	TGATTTCAAGGGAAGCAGAG	TGGTTTTCAAGCAGACAATCC	GAGGATGCTGCCATTTGTG
hCT2257127-Ex85	TGTAGAAAGCAAGGCTGCTC	TCCTCCTCAATGAAAGCAGAG	CATGCTAACAGAGTGTCAAGAGC
hCT1951422-Ex19	ACCCCAAAGTCATCCAAGTG	CAATGTGATCCCAACTGGTC	CGAATTCTTTTTGCCATTTC
hCT1951422-Ex20	AAAGGCTCCAGTTGATGGAC	TTATTGCCAATTGGAGTTTGG	AAAGTCTGCAAGGGGCTATG
hCT1951422-Ex21	CCATTAAAACCACTCTAAGTCAGG	TTCTGTTGGCTTATCATTTTTG	TCAGGCTAGAAATGTATCCAAGG
hCT1951422-Ex22	AAGCCTCCTCCAGAAAAGAAG	CCCAGAAACTAAATAAAATGCAG	AAAGGAAAGGGGTAATCCAG
hCT1951422-Ex23	CCCTCCTGTCCACTGAGATG	AATCAAATTTGTTGCATTAAAAATC	TTTACTTTTTATGATTACCTCTGATGC
hCT1951422-Ex24	TCTCAAGCTGCCTCACAATG	GTTTTCTCATTCCTTTCTCTTCC	AAAGAAAATTCAAATGAAAATAAGTCG
hCT1951422-Ex25	AAAGACATTGCCATGCAAAC	TTTGGGAAAGGGAACACAAG	CATGCAAACTTGGGTCTAGATG
hCT1951422-Ex26	TTGTTGGGCTCCAAATAAAC	GATTTTTCCTTGGAACATCCTC	TTGGCTTTTTCCCCTCATAC
hCT13051-Ex5	CCCTGGAGTGCTTACATGAG	CGGGGATCAGATTTGCTATG	TAAAGCCTTTCCCAGCTCAG
hCT13051-Ex6	GACTTTATAAACACTCGACATTAGAGC	TAGGGGGTCATCCTCAGGTC	CCTGCTGCTTCCACAGGAC
hCT13051-Ex7	ATGATGACCTCTGGCAGGAC	GTCTTCCCCTGCTCAATCAC	CATGGACGTCCTGTGGAAG
hCT13051-Ex8	GAATCAACCGTCAGCGTGTC	GACACGTTGTGGGCCAGCCAGT	GTGTCCCATTCATCCTCACC
hCT13051-Ex9	CTGGCACCGGGGAAAACAGAG	CTGCCGGTTATCTTCGGACACGTT	AACAGAGGAGGCGCTGAAG
hCT2282983-Ex40	TGGACATCGACTACAAGTCTGG	TGAGTGAGGGCAGACAGATG	GCCTCACCCTACCCATCC
hCT2282983-Ex41	TCCTTGGGGTTTTGAAGAAG	TGGCACCTGAACCATGTAAG	AGATTGCTGGGGTTCCTTTC
hCT2282983-Ex42	AAGGCCTTCCAGACTCTTGC	CGTACATGCCGAAGTCTGTC	CCACCTCACTCCATCTCTGG
hCT2282983-Ex43	CCTCTTTGTTTTTCCCTACCG	GCCCTGGTTTTAACCCTTAAC	TGGGGTAAGTTCCCTGAGTG
hCT2282983-Ex 44- 1	CTTCCACAGTGGGGGTACAG	CCAGCTCCAGCTTCTGACTC	TACAGAGCCAGGGAGAGTGC
hCT2282983-Ex 44- 2	GACACAACGGCAACATTATGCTG	TTGTGTTTTCTTGGAGACAG	TATCATCCACATCGGTCAGC

Table 2. Primers used for PCR amplification and sequencing

hCT2292935-Ex46	CATTCCAAAGCATCTGGTTTTAC	CAATGAGCATGGGAGAGATG	TTTGGGACAAGTAATTGTTATTAGC
hCT2292935-Ex47	TTGTGAGGAACGTGTGATTAGG	TGGAGTTTCTGGGACTACAGG	TTGAATGCAGTGGTGCTCTC
hCT2292935-Ex48	CTGGGCAACAGAGCAAGAC	CCTTCTTCAAAGCTGATTCTCTC	TCTGCCTGTGTTCTGAGCTG
hCT2292935-Ex49	TCCCTTCTCCTTTGGCTATG	CGCTCTACAGCCAATCACAG	GAACTCAGCTCTGCCTGGAC
hCT2292935-Ex50	ATAGCACCACTGCCTTCCAG	TGGCATCACAATCAATAGGG	GCGAGACTCGGTCTCAAAAG
hCT2292935-Ex51	TGCAGAAGTGGAGGTGGAG	CTCCAAGGGGGTTAGAGTCC	ATCGTTTGCCAACTCCTAGC
hCT2292935-Ex52	AACCCAAGCTGCTTCCTTTC	CAGGAAACCAGGTCAGAAGTG	AATCAGTGCAGGTGATGCAG
hCT2292935-Ex53	AGTCCTGCCCTGATTCCTTC	TTTTTGCAGAAAGGGGTCTTAC	ACATGGCCTGTGTCTGCTTC
hCT2292935-Ex54	CCCACCCACTTATTCCTGAG	GCCCACCCCACTCTAGAAAC	GACTGGAAGAAAATAACCAAGTTTC
hCT2292935-Ex55	TTTCCCCTTTAGGGTAGGTAGG	TGGAACCTTTTCTGCTCAAAG	GGCAGGCGTTAAAGGAATAG
hCT2292935-Ex56	CGGACATAGAGGAAGGATTGC	AGCTGCATGGTGCCAAAG	AAAAACAGGGCACCCATTG
hCT2292935-Ex57	TGGCCAAACTTTTCAAATCC	ATAACAATGGGCACATGCAG	TTAAGCCCACAGGGAACAAG
hCT2292935-Ex 58- 1	TGGGAGAGCTCAGGGAATAC	GGTCATTCTTCCATCAGCAAG	TGTCAGACCTTGGCCTTTTC
hCT2273636-Ex 35- 1	TCCCAAAGTGCTGGGATTAC	CACACCCACACTCACACAAAG	TCTTCTGAAAAATGGAGGAAGTC
hCT2273636-Ex 35- 2	TTGGCTGCCATGACTAACAC	GGCACTGCAGGCTAATAATG	GCTCTTCCTGGGGAAGTCTC
hCT2273636-Ex 36- 1	GCTCTCAGTGTGCCTCATGG	GGGACCTCAAGTCTTTTCCTTC	CAGTTTTTGACTGCCACTGC
hCT2273636-Ex 36- 2	AAGAAACACCCCGGTTCC	GGGACCTCAAGTCTTTTCCTTC	TCCATGCTCGACACTATTCTG
hCT2273636-Ex 37- 1	AAATTTAGTTGAGTAATGAGAGAATGC	GGAAGGGAAGGAGGACAAAC	TTCTACTTTACATACAAAAGGCACTC
hCT2273636-Ex 37- 2	GTAAAATTGGCCCTGCTTTG	CGTCTCAAACTACCAAGTCTGG	AGTTGGGCTTAGCCTGGATG
hCT2273636-Ex38	CATAACCACATGCAGCAACC	CACCCAGTGCTGTTTCAATG	AGTATCACGTCCATGTTGGAG
hCT2273636-Ex39	AATTGGCCTTGGAGACAGAC	CGCCGCATAATGTGTAAAAC	CAATGTTTGCTTTGAAAAAGG
hCT2273636-Ex 40- 1	TTCATGTGAGCAGGTATGCTG	TGCCATATTTAACTGCCATTTC	TGAGCAAAACCTGTGGAATG
hCT2273636-Ex 40- 2	TTGTGTACGACCCTCTGGTG	TGCCATATTTAACTGCCATTTC	TTTGCTGGTGCTGTCTATGG
hCT2273636-Ex41	TTTGTACAGTGGAGGCAACG	GCAGTCACTGAGACAGCTTTTATC	GGATGTGCAAAATGTTCTTCTG
hCT7084-Ex17	CAGCTGGTTATGTGTGTTTATGG	TAAGCATAGCCTCGGAGAAC	GGGAGCAGGTGTTATTGATTG
hCT7084-Ex18	TGTCCTCATGGTTGCTTTTC	GGACCATTAATAGCTACCTTCCTG	GGTGAGGAGTTTTCCCAAGC
hCT7084-Ex19	CAGGGACATGCTATCCAAAG	AGGCAAGACAACATATTTGAAAG	AGCACAGAGTTTGTTAATGTTTTTAG
hCT7084-Ex20	TGGTGGAACTTGTGTTTTTCC	AAGGGCTATGTGTCATTTTGTTC	GCTGACTTCTATTGGGAGCATAC
hCT7084-Ex21	TCATACGGTTTTGGCAGCTC	CATCAAGCAAGCAAACAAATG	CAGAGGTATGGTTTGGGTCTC

Table 2. Primers used for PCR amplification and sequencing

hCT7084-Ex22	ACAGAGGGAGAAGGGCTCAG	AATTCCCCCAAAAGCTTCC	TGGGGGTCTAGGACTATGGAG
hCT7084-Ex23	TGGGACAATTTTCGCAGAAG	TTCCCTCCTGGCTAAGAACC	GCTGTGTTTTCTTAATTTCCTGTATG
hCT7084-Ex 24- 1	ATGAAGCATGCTGCCTGATG	AAAAGCAGAGGGAATCATCG	CAGCCTCCTGCAGACTTTG
hCT2257641-Ex 1- 56	GGGGGCCTTTAGAAGGAAG	TCCCATTCATGACCTGGAAG	CATTTTGGGAAAGGAGGTTC
hCT2257641-Ex 1- 57	TGGAGTTCCTGAGAAATGAGC	GGCCCGCTTTAAGAGATCAG	CGGTCAGTATGACGGTAGGG
hCT2257641-Ex 1- 58	AGAGGGAACACCCTTTCCTG	CATGCCCAAAGTCGATCC	AGGTCATGAATGGGATCCTG
hCT2257641-Ex 1- 59	CATGATGTTGGAGCTTACATGC	ACACATCCATGGTGTTGGTG	GGCGCTAATCGTACTGAAAC
hCT2257641-Ex 1- 60	CGGGATTGGAGACAGACATC	TGCCACAGCCACATAGTCTC	TATGGTGGCCATGGAGACTG
hCT2257641-Ex 1- 61	CATCATGGTACACGCACTCC	TTCTATCTGCAGACTCCCACAG	AGGAGCCCTCCTTTGATTG
hCT29277-Ex55	CTCAATCAGAGCCTGAACCAC	GGAAAAGAAAGCAGGAGAAGC	GGCCAGTGGTATCTGCTGAC
hCT29277-Ex56	CCCGGCCTAAAGTTGTAGTTC	AAATGGAGAAAAGCCTGGTTC	AAGACAAAATCCCAAATAAAGCAG
hCT29277-Ex57	TGGGAGACTGTCAAGAGGTG	AAGCAATCCTCCCACCTTG	ATTGGTTTGAGTGCCCTTTG
hCT29277-Ex58	TTCCTCCAAGGAGCTTTGTC	CCTTCCTTTTTCACTCACACAC	AAAATGCTTTGCACTGACTCTG
hCT29277-Ex59	TTCCCTGTCCAGACTGTTAGC	TGATTTAATAATGAAGATGGGTTGG	TTCATCTTTATTGCCCCTATATCTG
hCT29277-Ex60	CCGGTTATGCACATCATTTAAG	ACTCAGTACCCCAGGCAGAG	TTAAAGATTATACCAAGTCAGTGGTC
hCT29277-Ex61	GCAGCCAGAGCAGAAGTAAAC	TCAAACTCCTGGGCTCAAAC	CATGTGGTTTCTTGCCTTTG
hCT29277-Ex62	TCTAATGAAAGCCCACTCTGC	CAGCCACATCCCCCTATG	AAGCATAGGCTCAGCATACTACAC
hCT29277-Ex63	AAGTGTGCATGATGTTTGTTCC	TGCCTTCTTCCACTCCTTTC	CCCATCAACTACCATGTGACTG
hCT29277-Ex 64- 1	GATGACCAAGAATGCAAACG	AAGAGTGAAAGCAGAGATGTTCC	GGTCCTGTTGTCAGTTTTTCAG
NM_005026 Ex17	ATCATCTTTAAGAACGGGGATGG	ACTAAGCCTCAGGAGCAGCCT	GGTCCTGGGGTGCTCCTAGA
NM_005026 Ex18	CCTCAGATGCTGGTGCCG	GATACTTGGGGAAGAGAGACCTACC	TCCTCAACTGAGCCAAGTAGCC
NM_005026 Ex19	TCTTCATGCCTTGGCTCTGG	GAGGGGAGAGGAGGGGGAG	TGTGTCCTCCATGTTCTGTTGG
NM_005026 Ex20	TCCGAGAGAGTGGGCAGGTA	CACAAACCTGCCCACATTGC	TGGCCCCTCTGCCTAGCA
NM_005026 Ex21	GGGCAGGTTTGTGGGTCAT	CCTGGGCGGCTCAACTCT	CCACTGCTGGGTCCTGGG
NM_005026 Ex22	GGAACTGGGGGCTCTGGG	AGGCGTTTCCGTTTATGGC	GAATAGAGAGCTTTTCCTGAGATGC
hCT1640694-Ex 1- 1	GTTTCTGCTTTGGGACAACCAT	CTGCTTCTTGAGTAACACTTACG	GATTCATCTTGAAGAAGTTGATGG
hCT1640694-Ex 1- 2	CTCCACGACCATCATCAGG	GATTACGAAGGTATTGGTTTAGACAG	ACTTGATGCCCCCAAGAATC
hCT1640694-Ex 1- 3	CCCCCTCCATCAACTTCTTC	GGTGTTAAAAATAGTTCCATAGTTCG	CTCAAGAAGCAGAAAGGGAAG
hCT1640694-Ex 2- 1	TCATCAAAAATTTGTTTTAACCTAGC	TATAAGCAGTCCCTGCCTTC	TCTACAGAGTTCCCTGTTTGC

Table 2. Primers used for PCR amplification and sequencing

hCT1640694-Ex 2- 2	TTCTGAACGTTTGTAAAGAAGCTG	TATAAGCAGTCCCTGCCTTC	GCTGTGGATCTTAGGGACCTC
hCT1640694-Ex 3- 1	GCAGCCCGCTCAGATATAAAC	CTGGGCGAGAGTGAGATTCC	AAAAAGCATTTCTGATATGGATAAAG
hCT1640694-Ex 3- 2	TCTGAAAATCAACCATGACTGTG	ATGAACCCAGGAGGCAGAG	TCGAAGTATGTTGCTATCCTCTG
hCT1640694-Ex 4- 1	TCTTGTGCTTCAACGTAAATCC	CGGAGATTTGGATGTTCTCC	AAAATAATAAGCATCAGCATTTGAC
hCT1640694-Ex 4- 2	TCTCAACTGCCAATGGACTG	CGGAGATTTGGATGTTCTCC	TTATTCCAGACGCATTTCCAC
hCT1640694-Ex5	TAGTGGATGAAGGCAGCAAC	TTTGTAGAAATGGGGTCTTGC	TTTGAGTCTATCGAGTGTGTGC
hCT1640694-Ex6	TGCCTTTTCCAATCAATCTC	AATTCCTGAAGCTCTCCCAAG	TTCCTGTTTTTCGTTTGGTTG
hCT1640694-Ex7	GGGGAAAAAGGAAAGAATGG	TGCTGAACCAGTCAAACTCC	TGAATTTTCCTTTTGGGGAAG
hCT1640694-Ex8	TTTGCTGAACCCTATTGGTG	TTGCAATATTGGTCCTAGAGTTC	TGGATCAAATCCAAATAAAGTAAGG
hCT1640694-Ex9	GATTGGTTCTTTCCTGTCTCTG	CCACAAATATCAATTTACAACCATTG	TTGCTTTTTCTGTAAATCATCTGTG
hCT1640694-Ex10	ACCTTTTGAACAGCATGCAA	TGGAAATAATGTTAAGGGTGTTTTT	TATTTCATTTATTTATGTGGAC
hCT1640694-Ex11	AAAACACCCTTAACATTATTTCCATAG	TCTGCATGGCCGATCTAAAG	GAAGTTAAGGCAGTGTTTTAGATGG
hCT1640694-Ex12	TTTATTCTAGATCCATACAACTTCCTTT	AAAGTTGAGAAGCTCATCACTGGTAC	ACCAGTAATATCCACTTTCTTTCTG
hCT1640694-Ex13	CTGAAACTCATGGTGGTTTTG	TGGTTCCAAATCCTAATCTGC	TTTATTGGATTTCAAAAATGAGTG
hCT1640694-Ex14	GAGTGTTGCTGCTCTGTGTTG	TTGAGGGTAGGAGAATGAGAGAG	TCTCATGTGAGAAAGAGATTAGCAG
hCT1640694-Ex15	GGATTCCTAAATAAAAATTGAGGTG	CATGCATATTTCAAAGGTCAAG	TGGCTTTCAGTAGTTTTCATGG
hCT1640694-Ex16	TTGCTTTCCTGAAGTTTCTTTTG	TCAAGTAAGAGGAGGATATGTCAAAG	CATGTGATGGCGTGATCC
hCT1640694-Ex17	GGGGAAAGGCAGTAAAGGTC	CATCAAATATTTCAAAGGTTGAGC	AGGAATACACAAACACCGACAG
hCT1640694-Ex18	TCCTTATTCGTTGTCAGTGATTG	GTCAAAACAAATGGCACACG	TGCACCCTGTTTTCTTTTCTC
hCT1640694-Ex19	CATGGTGAAAGACGATGGAC	TTACAGGCATGAACCACCAC	TGGACAAGTAATGGTTTTCTCTG
hCT1640694-Ex 20- 1	TGGGGTAAAGGGAATCAAAAG	CCTATGCAATCGGTCTTTGC	TGACATTTGAGCAAAGACCTG
hCT1640694-Ex 20- 2	TTGCATACATTCGAAAGACC	GGGGATTTTTGTTTTGTTTTG	TTTGTTTTGTTTTGTTTTTT

[1]SEQ ID NO: 6 to 165 (forward primers)
[2]SEQ ID NO: 166 to 325 (reverse primers)
[3]SEQ ID NO: 326 to 485 (sequencing primers)

Example 2—This example demonstrates the striking clustering of mutations within the PIK3CA gene

[36] All coding exons of PIK3CA were then analyzed in an additional 199 colorectal cancers, revealing mutations in a total of 74 tumors (32%) (Table 3 and examples in Figure 1).

Table 3 . *PIK3CA* mutations in human cancers

PIK3CA mutations*				Tumor type#								
Exon	Nucleotide	Amino acid	Functional domain	Colon	GBM	Gastric	Breast	Lung	Pancreas	Medullo-blastomas	Adenomas	Total
Exon 1	C112T	R38C	p85	1								1
Exon 1	G113A	R38H	p85	2								2
Exon 1	G263A	R88Q	p85	1								1
Exon 1	C311G	P104R	p85	1								1
Exon 1	G317T	G106V	p85	1								1
Exon 1	G323C	R108P	p85	1								1
Exon 1	del332-334	delK111		1								1
Exon 2	G353A	G118D		1								1
Exon 2	G365A	G122D		1								1
Exon 2	C370A	P124T		1								1
Exon 4	T1035A	N345K	C2	1								1
Exon 4	G1048C	D350H	C2		1							1
Exon 5	T1132C	C378R	C2		1							1
Exon 7	T1258C	C420R	C2	2								2
Exon 7	G1357C	E453Q	C2	1								1
Exon 9	C1616G	P539R	Helical	1								1
Exon 9	G1624A	E542K	Helical	9							1	10
Exon 9	A1625G	E542G	Helical	1								1
Exon 9	A1625T	E542V	Helical								1	1
Exon 9	G1633A	E545K	Helical	21				1				22
Exon 9	A1634G	E545G	Helical	1								1
Exon 9	G1635T	E545D	Helical	1								1
Exon 9	C1636A	Q546K	Helical	5								5
Exon 9	A1637C	Q546P	Helical	1								1
Exon 12	C1981A	Q661K	Helical	1								1
Exon 13	A2102C	H701P	Helical		1							1
Exon 18	G2702T	C901F	Kinase	1		1						2
Exon 18	T2725C	F909L	Kinase	1								1
Exon 20	T3022C	S1008P	Kinase	1								1
Exon 20	A3073G	T1025A	Kinase	1								1
Exon 20	C3074A	T1025N	Kinase	1								1
Exon 20	G3129T	M1043I	Kinase	2								2
Exon 20	C3139T	H1047Y	Kinase	2								2
Exon 20	A3140G	H1047R	Kinase	15		2	1					18
Exon 20	A3140T	H1047L	Kinase	1								1
Exon 20	G3145A	G1049S	Kinase		1							1
Tumors with mutations				74	4	3	1	1	0	0	2	
No. samples screened				234	15	12	12	24	11	12	76	
Percent of tumors with mutations				32%	27%	25%	8%	4%	0%	0%	3%	

*Exon number with nucleotide and amino acid change resulting from mutation. Nucleotide position refers to position within coding sequence, where position 1 corresponds to the first position of the start codon. Functional domains are described in Fig. 1 legend. #Number of non-synonymous mutations observed in indicated tumors. Colon, colorectal cancers; GBM, glioblastomas; gastric, gastric cancers; breast, breast cancers; lung, lung cancers; pancreas, pancreatic cancers; medulloblastomas; adenomas, benign colorectal tumors. All mutations listed were shown to be somatic except for five colorectal cancers and one glioblastoma where no corresponding normal tissue was available. Mutations were identified in 58 of 201 mismatch repair (MMR) proficient colorectal cancers, and 16 of 33 MMR-deficient colorectal cancers. Some tumors with *PIK3CA* mutations contained mutations in *KRAS* or *BRAF* while others did not, suggesting that these genes operate through independent pathways. Seven tumors contained two somatic alterations. In addition to the 92 nonsynonymous mutations recorded in the table, we detected 3 synonymous alterations.

Example 3—This example demonstrates that the mutations in PIK3CA occur late in tumorigenesis.

[37] To determine the timing of PIK3CA mutations during neoplastic progression, we evaluated 76 pre-malignant colorectal tumors of various size and degree of dysplasia. Only two PIK3CA mutations were found (E542K and E542V), both in very advanced adenomas greater than 5 cm in diameter and of tubuluvillous type. These data suggest that PIK3CA abnormalities occur at relatively late stages of neoplasia, near the time that tumors begin to invade and metastasize.

Example 4—This example demonstrates that PIK3CA mutations in a variety of different cancer types.

[38] We then evaluated PIK3CA for genetic alterations in other tumor types (Table 1). Mutations were identified in four of fifteen (27%) glioblastomas, three of twelve (25%) gastric cancers, one of thirteen (8%) breast, and one of twenty four (4%) lung cancers. No mutations were observed in eleven pancreatic cancers or twelve medulloblastomas. In total, 89 mutations were observed, all but 3 of which were heterozygous.

Example 5—This example demonstrates the non-random nature of the genetic alterations observed.

[39] The sheer number of mutations observed in PIK3CA in five different cancer types strongly suggests that these mutations are functionally important. This conclusion is buttressed by two additional independent lines of evidence. First, analysis of the ratio of non-synonymous to synonymous mutations is a good measure of selection during tumor progression, as silent alterations are unlikely to exert a growth advantage. The ratio of non-synonymous to synonymous mutations in PIK3CA was 89 to 2, far higher than the 2:1 ratio expected by chance ($P<1x10^{-4}$). Second, the prevalence of non-synonymous changes located in the PI3K catalytic and accessory domains was ~120

per Mb tumor DNA, over 100 times higher than the background mutation frequency of nonfunctional alterations observed in the genome of cancer cells ($P<1x10^{-4}$) (*9*).

[40] Although the effect of these mutations on kinase function has not yet been experimentally tested, their positions and nature within PIK3CA imply that they are likely to be activating. No truncating mutations were observed and >75% of alterations occurred in two small clusters in exons 9 and 20 (Table 2 and Figure 1). The affected residues within these clusters are highly conserved evolutionarily, retaining identity in mouse, rat, and chicken. The clustering of somatic missense mutations in specific domains is similar to that observed for activating mutations in other oncogenes, such as RAS (*10*), BRAF (*11, 12*), β-catenin (*13*), and members of the tyrosine kinome (*14*).

[41] These genetic data suggest that mutant PIK3CA is likely to function as an oncogene in human cancers.

Example 6—This example demonstrates that gene amplification of PIK3CA is not common.

[42] Quantitative PCR analysis of PIK3CA in 96 colorectal cancers showed no evidence of gene amplification, suggesting that gene copy alterations are not a significant mechanism of activation in this tumor type. The primers used were:

Real time PI3K hCT1640694 20-1F (intron)

TTACTTATAGGTTTCAGGAGATGTGTT (SEQ ID NO: 486); and

Real time PI3K hCT1640694 20-1R

GGGTCTTTCGAATGTATGCAATG (SEQ ID NO: 487)

[43] The Sequence Listing appended to the end of this application contains the following sequences:

SEQ ID NO: 1=coding sequence only (nt 13 to 3201 of SEQ ID NO: 2)
SEQ ID NO: 2=mRNA sequence (NM_006218)
SEQ ID NO: 3=protein sequence (NP_006209)
SEQ ID NO: 4=exon 9
SEQ ID NO: 5=exon 20
SEQ ID NO: 6 to 165 =forward primers
SEQ ID NO: 166 to 325=reverse primers
SEQ ID NO: 326 to 485=sequencing primers
SEQ ID NO: 486 and 487 amplification primers

References and Notes

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7. Catalytic subunits of PI3Ks were identified by analysis of InterPro (IPR) PI3K domains (IPR000403) present within the Celera draft human genome sequence. This resulted in identification of 15 PI3Ks and related PI3K genes. The kinase domain of PIK3CD gene was not represented in the current draft of human genome sequence and was therefore not included in this study.

8. Sequences for all annotated exons and adjacent intronic sequences containing the kinase domain of identified PI3Ks were extracted from the Celera draft human genome sequence (URL address: www host server, domain name celera.com). Celera and Genbank accession numbers of all analyzed genes are available in Table 1. Primers for PCR amplification and sequencing were designed using the Primer 3 program (URL address: http file type, www-genome.wi.mit.edu host server, cgi-bin domain name, primer directory, primer3_www.cgi subdirectory), and were synthesized by MWG (High Point, NC) or IDT (Coralville, IA). PCR amplification and sequencing were performed on tumor DNA from early passage cell lines or primary tumors as previously described (*12*) using a 384 capillary automated sequencing apparatus (Spectrumedix, State College, PA). Sequence traces were assembled and analyzed to identify potential genomic alterations using the Mutation Explorer software package (SoftGenetics, State College, PA). Of the exons extracted, 96% were

successfully analyzed. Sequences of all primers used for PCR amplification and sequencing are provided in Table S1.

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